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METHOD FOR DETECTING NUCLEIC ACIDS AND IMMOBILIZATION OF NUCLEIC ACIDS

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(57) Claim

1. Method for the detection of an analyte nucleic acid comprising the following steps:

   - hybridizing a detectably-labelled nucleic acid or a detectably-labelled nucleic acid hybrid with an immobilizable nucleic acid probe;

   - immobilizing the hybrid which forms and

   - detecting the amount of immobilized hybrid by means of the amount of label,

   wherein the immobilizable nucleic acid probe contains two or more nucleotides modified by immobilizable groups which are at least separated by 10 nucleotides in the nucleotide sequence.

2. Method as claimed in claim 1, wherein the distance between the modified nucleotides is between 11 and 40 nucleotides.

3. Method as claimed in claim 1 or 2, wherein the modified nucleotides represent the terminal nucleotides of the nucleic acid probe.

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COMPLETE SPECIFICATION

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INVENTION TITLE:

Immobilization of nucleic acids

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

The invention concerns a method for detecting nucleic acids, the use of special nucleic acids to immobilize nucleic acids and a system for the detection of nucleic acids.

The immobilization of nucleic acids is important in many areas for example when separating nucleic acids from other materials, when isolating nucleic acids with a special nucleotide sequence from a nucleic acid mixture and in addition for the detection of nucleic acids (nucleic acid diagnostics). In all these areas utilization is made of the special affinities of nucleic acids to those nucleic acids which have a nucleotide sequence which is essentially complementary to them and can hybridize under certain conditions with the desired nucleic acids. The complementary nucleic acids which are used for this which are denoted capture probe in the following are usually bound to a solid phase e.g. a membrane, a gel, a bead or a tube. Recently it has been established that the immobilization of nucleic acids to solid phases via covalently bound capture probes has drawbacks in several respects e.g. the rate of immobilization is reduced. Therefore it was suggested that the capture probe be specifically bound to the solid phase via affinity binding. Such a binding for example via antibodies/haptens or biotin/(strept-) avidin) enables a hybridization of the nucleic acid with the capture probe in a liquid and subsequent

immobilization of the hybrid formed. Such a procedure is described for example in EP-A-0 139 489.

The object of the present invention was to increase the effectiveness of the immobilization in order, in particular, to obtain more sensitive tests for nucleic acids.

The invention concerns a method for the detection of an analyte nucleic acid which comprises the following steps:

- Hybridization of a detectably labelled nucleic acid or of a detectably labelled nucleic acid hybrid with an immobilizable nucleic acid probe;

- immobilization of the hybrid formed and

- detection of the amount of immobilized hybrid via the amount of label,

  wherein the immobilizable nucleic acid probe contains two or more nucleotides modified by immobilizable groups which are not directly adjacent in the nucleotide sequence.

More particularly, the present invention contemplates a method for the detection of an analyte nucleic acid comprising the following steps:

- hybridizing a detectably-labelled nucleic acid or a detectably-labelled nucleic acid hybrid with an immobilizable nucleic acid probe;

- immobilizing the hybrid which forms and

- detecting the amount of immobilized hybrid by means of the amount of label,

wherein the immobilizable nucleic acid probe contains two or more nucleotides modified by immobilizable groups which are at least separated by 10 nucleotides in the nucleotide sequence.

The invention also concerns the use of the nucleic acids mentioned in the method as well as a system for detecting nucleic acids.

An analyte nucleic acid within the sense of the invention is a nucleic acid which is to be detected. It is usually present in a sample which also contains further components, for example a tissue or a liquid. In

particular there are also additional nucleic acids in the sample which are not intended to be detected. The nucleic acid can be any type of nucleic acid, for example DNA, RNA or fragments thereof. If the analyte nucleic acid is originally present in the sample in a double-stranded form it is preferably converted into the single-stranded form. If the analyte nucleic acid is present bound to a solid phase it is preferably brought into solution.

A detectably labelled nucleic acid is understood as a nucleic acid whose presence or amount can be determined with the aid of a detection system. For this purpose a detectably labelled nucleic acid has modifications as compared to a normal nucleotide sequences. Suitable modifications are familiar to one skilled in the art. An example of a modification are chemical groups which are not normally present in nucleic acids such as enzymes, coloured or fluorescent molecules and ligands, preferably residues capable of binding immunologically. Examples of the latter are in particular haptens such as digoxigenin and also vitamins such as biotin. In the case of enzymes the detection is usually carried out via a colour-forming reaction catalyzed by the enzyme. Coloured or fluorescent molecules can be detected directly by photometric or fluorometric means. Ligands are usually contacted with a corresponding enzyme-labelled or dye-labelled receptor which has affinity to the ligands and are detected by this means.

A detectably labelled nucleic acid hybrid is understood as a partially double-stranded hybrid of at least two nucleic acids. In this case either both nucleic acids or only one of them may be detectably labelled as described above. It is important that the nucleic acid hybrid has

a single-stranded region. In a so-called sandwich test the hybrid preferably consists of the (non-labelled) analyte nucleic acid and a detectably labelled detector probe.

An immobilizable nucleic acid probe is understood as a nucleic acid probe which is modified by a residue which is not present in the usual nucleic acids. Such residues are residues which have an affinity to another material. Examples are partners of a biospecific reaction between a ligand and a receptor for example an immunological reaction, a reaction between sugar and lectin or between vitamin and binding protein. Particularly preferred residues are haptens or biotin. In the method according to the present invention the residue on the immobilizable nucleic acid probe is different from the detectable residue of the detectably labelled nucleic acid.

The steps of the method of detection described in the following are known as individual steps or can be carried out analogous to known methods. Reference is in particular made to Molecular Cloning, Editor Sambrook et al., CSH 1989. These also include the known methods for the production of labelled nucleoside triphosphates, the chemical synthesis of modified and unmodified oligonucleotides, the choice of hybridization conditions by which means a specificity can be achieved which is dependent on the extent of homology between the nucleic acids to be hybridized, their GC content and their length, as well as the formation of nucleic acids from nucleoside triphosphates with the aid of polymerases and if desired, using so-called primers. An essential feature of the present invention is the use of a special capture probe.

In a first step of the method according to the present invention a detectably labelled nucleic acid or a detectably labelled nucleic acid hybrid is produced using the analyte nucleic acid. This can for example be carried out by detectably labelling the analyte nucleic acid itself. This can be achieved by the enzymatic incorporation of labelled nucleoside triphosphates or by elongating the analyte nucleic acid with labelled nucleoside triphosphates (tailing). A further possibility is to amplify a region of the analyte nucleic acid while incorporating detectably labelled mononucleoside triphosphates or detectably labelled oligonucleotides. Many methods are known for this from the state of the art.

One possibility is to carry out an amplification by means of the polymerase chain reaction according to EP-A-0 200 362 using the analyte nucleic acid as the template nucleic acid. In this case two primers are used one of which is complementary to a region of the analyte nucleic acid and the other is complementary to a part of the opposite strand of the analyte nucleic acid to form a multitude of copies of the strand and opposite strand of the analyte nucleic acid by elongation of the primer on the template. In order to produce the detectably labelled nucleic acid, at least one detectably labelled mononucleoside triphosphate is used in the elongation of the primer or a labelled primer is used.

A further possibility arises from the use of the ligase chain reaction according to WO 90/06376 if at least one of the oligonucleotides is detectably labelled.

A further possibility is described in EP-A-0 329 822. In this process a DNA strand is formed which is

complementary to substantial parts of the analyte nucleic acid and a primer containing a promoter is incorporated. After degradation of the analyte nucleic acid, a transcribable double strand is formed which can be used to form a multitude of detectably labelled transcripts while incorporating labelled mononucleoside triphosphates.

The method according to the present invention has proven to be particularly efficient when in the form of a sandwich nucleic acid assay e.g. according to EP-A-0 192 168. The method according to the present invention differs from the known sandwich assays in that a particular type of capture probe is used.

In a further step the detectably labelled nucleic acid or the detectably labelled nucleic acid hybrid is brought into contact with an immobilizable nucleic acid probe under hybridization conditions.

The immobilizable nucleic acid probe according to the present invention contains two or more nucleotides modified by immobilizable groups which are not directly adjacent in the nucleotide sequence. Apart from the number of ligands (reporter groups) the distance between the ligands is crucial and should be preferably 10 or more nucleotides in order to observe this effect. For example oligodeoxyribonucleotide probes which contained 5 biotin residues (biotin corresponds to the ligand) coupled in direct succession and were used as capture probes to bind an analyte to a streptavidin matrix did not result in an increase in the sensitivity in the total assay compared to a reference probe which was only linked to one biotin residue (see example 2). The "capture probe" can be an oligonucleotide or a

polynucleotide (DNA or RNA) which can hybridize in a suitable manner with the analyte nucleic acid to be detected. It can be single-stranded (e.g. oligodeoxyribonucleotide, oligoribonucleotide) or double-stranded (e.g. plasmid, fragment). In the latter case the capture probe has to be denatured before hybridization with the analyte. A particularly preferred embodiment utilizes nucleic acid probes in which the terminal nucleotides of the nucleic acid probe are modified in each case.

An oligodeoxynucleotide is preferred which has a length between 11 and 40 nucleotides whereby the label is appropriately attached to the 3'- and 5'- end in order to meet the requirement for distance as described above.

The immobilizable residues can in principle be bound to the base part or to the sugar part or to the phosphate part of the nucleotides. Such immobilizable nucleic acid probes can be produced according to known methods or analogous to known methods. The attachment of a ligand to nucleotides with modified bases is described for example in Nucleic Acids Res. 15 (12), p. 4857 to 4876 (1987); Nucleic Acids Res. 16 (9), p. 4077 to 4095 (1988); Nucleic Acids Res. 17, p. 4643 to 7650 (1989) or can be carried out by introducing a modified nucleotide which already contains the ligand (e.g. Nucleic Acids Res. 18 (15), p. 4355 to 4360 (1990). A further suitable chemical method for incorporating several primary amino groups in synthetic oligonucleotides which can then be used for binding to an immobilizable residue is described in Nucleic Acids Res. 17 (18), p. 7179 to 7194 (1989). The attachment of the ligand to a nucleic acid can also be carried out by a bisulfide-catalyzed transamination (Nucleic Acids Res. 12 (2), p. 989 to

1002 (1984)). The attachment of several immobilizable residues can also be carried out by enzymatic methods e.g. by tailing using a nucleoside triphosphate containing the appropriate residue (DNA 5 (4), p. 333 to 337 (1986)) or by random priming (Analyt. Biochem. 132, p. 6 1983).

Chemically synthesized oligonucleotides are particularly preferred within the scope of the present invention since they have the advantage that the distance between two modified nucleotides can be exactly determined beforehand.

The use of multiply labelled nucleic acids as detection probes was known previously. For example in EP-A-0 330 221 an oligonucleotide or a polynucleotide is labelled with biotin and subsequently biotin is detected by means of a streptavidin-enzyme complex. The separation of two biotin-dUMP residues in the nucleotide sequence by simultaneous incorporation of dTTP is described as being more of a disadvantage. In Nucleic Acids Res. 18 (15), p. 4358 it is shown that by attaching several immobilizable residues to nucleic acids their binding to solid phases can be improved.

In Nucleic Acids Res. 18, p. 4345 to 4354 (1990) multiply labelled nucleic acids are also used as a detection probe. The production of multiply labelled nucleic acids in which the label is attached to the phosphate residue is described in WO 90/08838.

If the sample contains analyte nucleic acid, a nucleic acid hybrid forms from the detectably labelled nucleic acid or the detectably labelled nucleic acid hybrid and

the immobilizable nucleic acid probe. In the method according to the present invention this is bound via the immobilizable residue to a solid phase. The surface of the solid phase contains groups which have an affinity to the immobilizable residue of the nucleic acid probe, for example a receptor. If the immobilizable group is a hapten, the solid phase preferably contains an antibody against this hapten. In the case of biotin the solid phase contains a biotin binding protein such as avidin or streptavidin.

The amount of immobilizable hybrid is a measure for the amount or the presence of the analyte nucleic acid. This amount can be detected via the amount of label which is immobilized on the solid phase. This is preferably carried out in a known manner and depends on the type of label used. Before carrying out the detection reaction, the solid phase is preferably removed from the liquid phase. This at the same time results in the removal of the starting material for the production of the detectably labelled nucleic acid or for the detectably labelled nucleic acid hybrid or of an excess detectably labelled nucleic acid probe in the case of a sandwich hybridization together with the liquid. The detection of a nucleic acid by the method according to the present invention, especially if the test is to be carried out quantitatively, encompasses comparing the measurement signal which was obtained with the sample of unknown analyte nucleic acid content with the measurement signal or measurement signals which were obtained with one or several samples with known analyte nucleic acid content. The use of a calibration curve is preferred which can also be provided in the form of entered data.

The invention in addition concerns the use of a nucleic acid which contains two or more nucleotides modified by immobilizable groups which are not directly adjacent in the nucleotide sequence for immobilizing nucleic acids. This aspect is based on the fact that the nucleic acid probes according to the present invention guarantee a surprisingly improved immobilization. The method can for example be used in the affinity separation of nucleic acids.

The invention also relates to a system for the detection of nucleic acids which contains one or several nucleic acids containing two or more nucleotides modified by immobilizable groups which are not directly adjacent in their respective nucleotide sequence and at least one solid phase which has a specific affinity to the immobilizable groups of the nucleic acid. Before starting a detection method it is preferable that the nucleic acids and the solid phase are present separated from one another in the system. In addition the system can also contain further components which are necessary or helpful for the detection of nucleic acids. These in particular include pH buffers and reagents for detecting the label.

Fig. 1 shows a diagram of the binding of an analyte nucleic acid A via a capture probe F according to the present invention to a solid phase coated with streptavidin S in a sandwich test. The detectably (represented by a rhombus) labelled detector probe is denoted D.

Fig. 2 shows the binding to the wall of a labelled analyte nucleic acid A' via a capture probe according to

the present invention to a solid phase coated with streptavidin S.

Fig. 3 illustrates the increase of the sensitivity of a sandwich nucleic acid test in which the immobilizable groups are located on adjacent nucleotides by separating the groups according to the present invention. It is clear that the increase in sensitivity when using only two biotin residues which are each at the ends of the oligonucleotide is unexpectedly even more than when 10 biotin residues are used at one end (see also example 2).

Fig. 4 shows the increase in sensitivity in the sandwich test when using two biotin residues compared to the use of one biotin residue with an oligonucleotide length of 30 nucleotides.

Fig. 5 shows the comparison of sensitivity of Figure 4, but with oligonucleotides having a length of 20 nt.

Fig. 6 shows the comparison of sensitivity for a method in which an analyte nucleic acid was amplified and at the same time labelled by PCR and in which nucleic acid probes with a length of 15 nucleotides and having one or two biotin residues were used as the capture probe.

The curves shown in figures 4 to 6 can also be used as calibration curves for determining an unknown amount of an analyte nucleic acid.

Fig. 7 shows the formula for the aminomodifier II.

The invention is elucidated in more detail by the following examples.

Example 1

Production of oligonucleotides

A: Explanatory notes

All oligonucleotides were produced with the aid of a DNA synthesizer 8700 from the Biosearch Company using the "phosphoramidite method" published by Caruthers et al. Methods Enzymol. 154, 287, 1987.

The β-cyanoethyl-nucleoside phosphoramidites used were obtained from Roth (Karlsruhe, GFR), D-biotinoyl-aminocaproic acid-N-hydroxysuccinimide ester was from Boehringer Mannheim (Mannheim, GFR), aminomodifier 2 (AM II) from Beckmann Instruments GmbH (Fullerton, CA, USA). All other reagents and solvents were used in the best quality available.

The purification of the biotinylated oligonucleotides was carried out by HPLC (Spectra Physics, Darmstadt, GFR) using a Mono Q HR 5/5 ion-exchange column from Pharmacia (Freiburg, GFR) or a C_{18} column (LiChrosorb RP 18-5, CS-Chromatographie Service, Langerwehe, GFR). Spectra/Por 1000 membranes (Roth, Karlsruhe, GFR) were used for the dialysis.

B) Synthesis and purification of the biotinylated oligonucleotides

The biotinylated oligonucleotides were all prepared in the Biosearch synthesizer on a 1 μmol scale using the trityl-off as well as cleave-off programme. Since there was no carrier material available with aminomodifier 2, a thymidine carrier was used and subsequently aminomodifier 2 (see Fig. 7) was condensed to this according to the standard coupling program (0.1 M in acetonitrile).

After the aminomodifier, a further thymidine was coupled to the 5' end in order to protect the primary hydroxyl group of the aminomodifier from attack during the ammonia treatment and the glyceryl residue from being again partially cleaved off. After completion of the synthesis and cleavage of the protecting groups with concentrated NH_3 solution at 55°C/5h, all solvents were removed in a vacuum, the residue was taken up in water and the product was purified by ion-exchange chromatography (Mono Q HR 5/5; A: 0.25 mM Tris/HCl, 0.3 M NaCl; B: 0.25 Tris/HCl, 1 M NaCl; in 60 min from 0 to 100 % B, flow 1 ml/min). Subsequently the oligodeoxynucleotide was desalted by dialysis (Spectra/Por 1000, Roth). Afterwards the oligonuclotides were biotinylated by taking up 5-10 $O.D._{260nm}$ of the oligodeoxynucleotides in 0.5 ml 0.05 M K_2HPC_4/KH_2PO_4 buffer and adding a solution of 5 mg D-biotinoyl-aminocaproic acid-N-hydroxysuccinimide ester in 0.5 ml DMF. The reaction mixture was incubated overnight at 37°C, subsequently the solvent was removed in a vacuum, the residue was taken up in redistilled water and

excess biotin was removed by filtration. The further purification was carried out by means of reversed phase HPLC with a gradient of A: 0.1 M triethylammonium acetate pH 7; 5 % acetonitrile, B: 0.1 M triethylammonium acetate pH 7; 40 % acetonitrile. The gradient was run from 20 % B to 80 % B within 40 min at a flow rate of 2 ml/min. The difference in the retention time between the twice biotinylated oligonucleotides and the corresponding aminomodifier-modified starting oligonucleotides was between 2 and 4 minutes depending on the length of the sequence. The biotinylated oligodeoxynucleotides could be obtained in 1.5 to 3.5 $O.D._{260}$ after renewed dialysis.

Example 2

Detection of HBV analyte DNA in a sandwich form taking into account the site of modification

The test is carried out in a sandwich with binding of the analyte DNA to a solid phase. A partial sequence of the hepatitis B virus DNA which is present cloned in a plasmid is used as the analyte DNA. The analyte DNA is bound to a streptavidin solid phase by means of a biotin-labelled oligonucleotide whose sequence is complementary to a region of the HBV DNA. The detection of the bound analyte DNA is then carried out by means of a digoxigenin-labelled oligonucleotide which in turn is complementary to another region of the analyte DNA. This can be recognized by anti-digoxigenin antibodies which are conjugated with horseradish peroxidase and the hybrid is subsequently detected by an enzyme-catalyzed colour-forming reaction.

A dilution series of the plasmid in a range of 4.4 µg/ml, 2.2 µg/ml, 1.1 µg/ml, 0.55 µg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) is prepared. In order to denature the double-stranded DNA, 10 µl 5 M NaOH is added to 90 µl of the plasmid solution and incubated for 10 min at room temperature. 20 µl of the denaturation preparation is subsequently pipetted into a well of a microtitre plate coated with streptavidin and immediately neutralized by addition of 180 µl hybridization solution (50 mM Na phosphate buffer, 0.75 M NaCl, 0.075 M Na citrate, 0.05 % bovine serum albumin, pH 5.4). This results in the following concentrations of the plasmid in the test: 50, 100, 200, 400 ng/ml. The hybridization solution in addition contains 200 ng/ml of a digoxigenin-labelled oligonucleotide (labelled once with digoxigenin at the 5' end, position 287c-248c in the HBV genome) and of a biotin-labelled oligonucleotide (position 2456c-2417c in the HBV genome). The test is carried out with oligonucleotides having different degrees of biotin labelling:

HBV-Oli 7-4 (1 biotin, 40mer), SEQ ID NO 1:
5'-T(bio-AMII)-CATTGAGATTCCCGAGATTGAGATCTTCTGCGACGCGGCG-3'

HBV-Oli 7-5 (5 biotin, 40mer), SEQ ID NO 2:
5'-T-(bio-AMII)5-
CATTGAGATTCCCGAGATTCAGATCTTCTGCGACGCGGCG-3'

HBV-Oli 7-6 (10 biotin, 40 mer), SEQ ID NO 3:
5'T-(bio-AMII)10-
CATTGAGATTCCCGAGATTGAGATCTTCTGCGACGCGGCG-3'

HBV-Oli 7-7 (2 biotin, 40mer), SEQ ID NO 4:
5'T-(bio-MII)-CATTGAGATTCCCGAGATTCAGATCTTCTGCGACGCGGCG-(AMII-bio)-T-3'

The hybridization preparation is incubated for 3 h at 37°C in the microtitre plate while shaking. After aspirating the solution it is washed 2 x 10 min with 0.3 M NaCl, 0.03 M Na citrate, 0.2 % Na dodecylsulfate at 37°C and subsequently once for a short time at room temperature with 0.9 % NaCl in order to remove non-bound reaction partners from the test. 20 mU/ml of an anti-digoxigenin antibody-horseradish peroxidase conjugate in 100 mM Tris-HCl (pH 7.5), 0.9 % NaCl, 1 % bovine serum albumin is added and incubated for 30 min at 37°C while shaking. Non-bound conjugate is removed by briefly washing three times with 0.9 % NaCl at room temperature. The detection reaction is started by addition of the substrate solution ABTS^R (1.9 mM, 2,2'azino.di-[3-ethylbenzthiazoline sulfonic acid (6)]-diammonium salt). The incubation is carried out for 30 min at 37°C while shaking. The absorbance is subsequently measured at 405 nm by means of an ELISA reader.

The results are shown in Fig. 3.

Example 3

Detection of HBV-analyte DNA in a sandwich format taking into account the chain length and the site of modification

A dilution series of the plasmid which contains HBV-specific sequences is prepared in a range from 62.5 to 250 ng/ml in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. 10 µl

5 M NaOH is added to 90 µl of the plasmid solution and incubated for 10 min at room temperature. 20 µl of this denaturation preparation is pipetted into a well of a microtitre plate coated with streptavidin and 180 µl hybridization solution (50 mM phosphate buffer, 0.75 M NaCl, 0.075 M Na citrate, 0.05 % bovine serum albumin (BSA), pH 5.4) is added. 200 ng/ml of the biotin-labelled capture oligonucleotide and of the digoxigenin-labelled oligonucleotides (pos. 405-444 in the HBV genome, 40mer, labelled twice with digoxigenin at the 3' and 5' end) are added to the hybridization solution. Thus the analyte DNA concentration in the test is 6.25, 12.5, 25 and 50 ng/ml.

HBV-Oli 25-3 (pos. 2327-2356, 30mer, 1 biotin),
SEQ ID NO 5:
5'-T-(bio-AMII)-CACTTCCGGAAACTACTGTTGTTAGACGAC-3'

HBV-Oli 25-4 (pos. 2327-2356, 30mer, 2 biotin),
SEQ ID NO 6:
5'-T-(bio-AMII)-CACTTCCGGAAACTACTGTTGTTAGACGAC-(AMII-bio-)-T-3'

HBV-Oli 33-1 (pos. 2332-2351, 20mer, 1 biotin).
SEQ ID NO 7:
5'-T-(bio-AMII)-CCGGAAACTACTGTTGTTAG-3'

HBV-Oli 33-2 (pos. 2332-2351, 20mer, 2 biotin),
SEQ ID NO 8:
5'-T-(bio-AMII)-CCGGAAACTACTGTTGTTAG-(AMII-Bio)-T-3'

The stated positions relate to the HBV genome.

The hybridization preparation is shaken for 3 h at 37°C. Subsequently it is washed 2 x 10 min with 0.3 M NaCl, 0.03 M Na citrate, 0.2 % Na dodecylsulfate at 37°C and then once briefly with 0.9 % NaCl at room temperature. Subsequently 200 µl of the anti-digoxigenin antibody-horseradish peroxidase conjugate (200 mU/ml in 100 mM Tris-HCl, pH 7.5, 0.9 % NaCl, 1 % BSA) is added and incubated for 30 min at 37°C while shaking. After washing again three times with 0.9 % NaCl, the substrate solution (1.9 mM ABTS^R) is pipetted shaken again for 30 min at 37°C and then the absorbance is measured at 405 nm by means of an ELISA reader.

The results for the 30 nt oligonucleotides are shown in Fig. 4, those for the 20 nt oligonucleotides are shown in Fig. 5.

Example 4

Detection of PCR products with singly- and twice-labelled oligonucleotides

For the DNA test for the hepatitis B viruses, the viral DNA is firstly amplified by means of the polymerase chain reaction (EP-A-0 200 362). During this amplification digoxigenin is incorporated into the DNA in the form of Dig-11-dUTP (EP-A-324474). Subsequently this hapten allows a sensitive detection of the PCR product in a heterogeneous immunoassay. The binding of the digoxigenin-labelled DNA to the walls of the streptavidin-coated tubes is carried out by means of a biotin-labelled oligonucleotide whose region is complementary to a region of the PCR product. The test is again carried out using an anti-digoxigenin antibody-

horseradish peroxidase conjugate and a subsequent colour reaction.

HBe-positive human plasma with a virus titre of 1 x 10^{10} hepatitis B viruses per ml is diluted in normal serum so that the virus content in the dilutions is 1 x 10^{7} viruses/ml. For the lysis, 10 µl 0.2 M NaOH is added to 10 µl of the virus dilution and incubated for 1 h at 37°C. The lysis preparation is neutralized by addition of 30 µl neutralization mixture (100 mM KCl, 50 mM Tris-HCl, pH 6.5, 3 mM $MgCl_2$). 20 µl of this solution is used in the subsequent amplification reaction (amplification conditions: 200 nM of each of the PCR primers, 200 µM each of dATP, dCTP, dGTP, 175 µM dTTP, 25 µM digoxigenin-11-2'-dUTP, 2.5 U Thermus aquaticus DNA polymerase in 50 mM KCl, 10 mM Tris-HCl, pH 8.9, 1.5 mM $MgCl_2$, 0.01 % gelatin; total volume 100 µl). The preparation is covered with a layer of 100 µl mineral oil and incubated for 30 cycles in a thermo-cycler (Perkin-Elmer):

30 sec 92°C, 30 sec 50°C, 60 sec 70°C.

Due to the position of the PCR primers (PCR primer 1: position 1937-1960; PCR primer 2: position 2434c-2460c in the HBV genome) a 500 bp long DNA fragment is produced which is subsequently detected in a two-component test system in streptavidin-coated microtitre plates.

The PCR preparation is diluted in H_2O in such a way that in relation to the original virus concentration of 1 x 10^{7} viruses per ml serum, dilutions are made which correspond to a range of 1 x 10^{5} to 1 x 10^{4} viruses/ml.

20 μl of the diluted PCR preparation is incubated in a final concentration of 0.1 N NaOH in a volume of 50 μl for 10 min at room temperature for the denaturation. 20 μl of the denaturation solution are pipetted together with 180 μl hybridization solution (1 M NaCl, 0.1 M Na citrate, 67.5 mM Na phosphate, 0.05 % BSA, pH 6.7) into a streptavidin-coated well of a microtitre plate. The biotin-labelled oligonucleotides are added at a concentration of 100 ng/ml to the hybridization buffer:

HBV-Oli 34-1 (pos. 2299-2313, 15mer, 1 biotin),
SEQ ID NO 9:
5'-T-(AMII-bio)-AGACCACCAAATGCC-3'

HBV-Oli 34-2 (pos. 2299-2313, 15mer, 2 biotin),
SEQ ID NO 10:
5'-T-(AMII-bio)-AGACCACCAAATGCC-(bio-AMIIH)-T-3'

The hybridization preparation is incubated for 3 h at 37°C while skaking. Subsequently the solution is aspirated and the well is washed three times with 0.9 % NaCl. 200 μl of a solution of 200 mU/ml anti-digoxigenin antibody-horseradish peroxidase conjugate in 100 mM Tris-HCl, pH 7.5, 0.9 % NaCl, 1 % BSA is added and incubated for 30 min at 37°C. After washing again with 0.9 % NaCl the substrate reaction is started by addition of 200 μl ABTS^R. The photometric measurement is carried out after 30 min at 405 nm.

The results are shown in Fig. 6.

Sequence protocol

SEQ ID NO 1

Length of sequence:	41 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2456c-2417c (Hpbadw-data bank)
Antisense	
Modification N:	T on this is bound to aminomodifier II via 3'-0 biotin

5'-N CAT TGA GAT TCC CGA GAT TGA GAT CTT CTG CGA CGC GGC G-3'

SEQ ID NO 2

Length of sequence:	41 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2456c-2417c
Antisense	
Modification N:	T on this is bound to 5 aminomodifiers II via 3'-0 5 biotin

5'-N CAT TGA GAT TCC CGA GAT TGA GAT CTT CTG CGA CGC GGC G-3'

SEQ ID NO 3

Length of sequence:	41 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2456c-2417c
Antisense	
Modification N:	T on this is bound to 10 aminomodifiers II via 3'-0 10 biotin

5'-N CAT TGA GAT TCC CGA GAT TGA GAT CTT CTG CGA CGC GGC G-3'

SEQ ID NO 4

Length of sequence:	42 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2456c-2417c
Antisense	
Modification N at the 5' end:	T on this is bound to aminomodifier II via 3'-0 biotin
Modification N at the 3' end:	T on this is bound to aminomodifier II via 5'-0 biotin

5'-N CAT TGA GAT TCC CGA GAT TGA GAT CTT CTG CGA CGC GGC N-3'

SEQ ID NO 5

Length of sequence:	31 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2327-2356
Modification N:	T on this is bound to aminomodifier II via 3'-O biotin

5'-N CAC TTC CGG AAA CTA CTG TTG TTA GAC GAC 3'

SEQ ID NO 6

Length of sequence:	32 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2327-2356
Modification N at the 5'end:	T on this is bound to aminomodifier II via 3'-O biotin
Modification N at the 3'end	T on this is bound to aminomodifier II via 5'-O biotin

5'-N CAC TTC CGG AAA CTA CTG TTG TTA GAC GAC N-3'

SEQ ID NO 7

Length of sequence:	20 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2332-2351
Modification N:	T on this is bound to aminomodifier II via 3'-O biotin

5'-NC CGG AAA CTA CTG TTG TTA G-3'

SEQ ID NO 8

Length of sequence:	21 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2332-2351
Modification N at the 5' end:	T on this is bound to aminomodifier II via 3'-0 biotin
Modification N at the 3' end:	T on this is bound to aminomodifier II via 5'-0 biotin

5'-NC CGG AAA CTA CTG TTG TTA GN-3'

SEQ ID NO 9

Length of sequence:	16 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2299-2313
Modification N:	T on this is bound to aminomodifier II via 3'-0 biotin

5'-N AGA CCA CCA AAT GCC-3'

SEQ ID NO 10

Length of sequence:	17 bases
Type of sequence:	nucleotide sequence
Type of strand:	single strand
Topology:	linear
Type of molecule:	part of genome DNA with modifications N
Position:	HBV genome 2299-2313
Modification N at the 5' end	T on this is bound to aminomodifier II via 3'-0 biotin
Modification N at the 3' end:	T on this is bound to aminomodifier II via 5'-0 biotin

5'-N AGA CCA CCA AAT GCC N-3'

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Method for the detection of an analyte nucleic acid comprising the following steps:

   - hybridizing a detectably-labelled nucleic acid or a detectably-labelled nucleic acid hybrid with an immobilizable nucleic acid probe;

   - immobilizing the hybrid which forms and

   - detecting the amount of immobilized hybrid by means of the amount of label,

   wherein the immobilizable nucleic acid probe contains two or more nucleotides modified by immobilizable groups which are at least separated by 10 nucleotides in the nucleotide sequence.

2. Method as claimed in claim 1, wherein the distance between the modified nucleotides is between 11 and 40 nucleotides.

3. Method as claimed in claim 1 or 2, wherein the modified nucleotides represent the terminal nucleotides of the nucleic acid probe.

4. Method as claimed in claim 1 or 2, wherein the immobilizable groups are bound to phosphate groups of the nucleotides.

5. Method as claimed in claim 4, wherein the immobilizable groups are bound to phosphate groups of the nucleotides.

6. Method as claimed in claim 1 or 2, wherein the immobilizable groups are bound to the base part of the nucleotides.

7. Method as claimed in claim 3, wherein the immobilizable groups are bound to the base part of the nucleotides.

8. Method as claimed in claim 1 or 2, wherein the detectably-labelled nucleic acid is an amplification product of a region of the analyte nucleic acid.

9. Method as claimed in claim 3, wherein the detectably-labelled nucleic acid is an amplification product of a region of the analyte nucleic acid.

10. Method as claimed in claim 1, 2, 5 or 7, wherein the detectably-labelled hybrid is a hybrid of the analyte nucleic acid and a detectably-labelled nucleic acid probe.

11. Method as claimed in claim 3, wherein the detectably-labelled hybrid is a hybrid of the analyte nucleic acid and a detectably-labelled nucleic acid probe.

12. Method as claimed in claim 4, wherein the detectably-labelled hybrid is a hybrid of the analyte nucleic acid and a detectably-labelled nucleic acid probe.

13. Method as claimed in claim 6, wherein the detectably-labelled hybrid is a hybrid of the analyte nucleic acid and a detectably-labelled nucleic acid probe.

14. Use of a nucleic acid containing two or more nucleotides modified by immobilizable groups which are at least separated by 10 nucleotides in the nucleotide sequence for immobilizing nucleic acids.

15. System for the detection of nucleic acids containing

- one or more nucleic acids which contain two or more nucleotides modified by immobilizable groups which are at least separated by 10 nucleotides in the respective nucleotide sequence and

- at least one solid phase which has a specific affinity for the immobilizable groups of the nucleic acid.

16. A method or system for detecting nucleic acids substantially as hereinbefore described with reference to the drawings and/or examples.

DATED this 3rd day of June, 1993

BOEHRINGER MANNHEIM GmbH
By Its Patent Attorneys
DAVIES COLLISON CAVE

Abstract

Method for the detection of nucleic acids in which a nucleic acid probe having two or more nucleotides modified by immobilizable group which are not directly adjacent in the nucleotide sequence is used.

FIG. 1

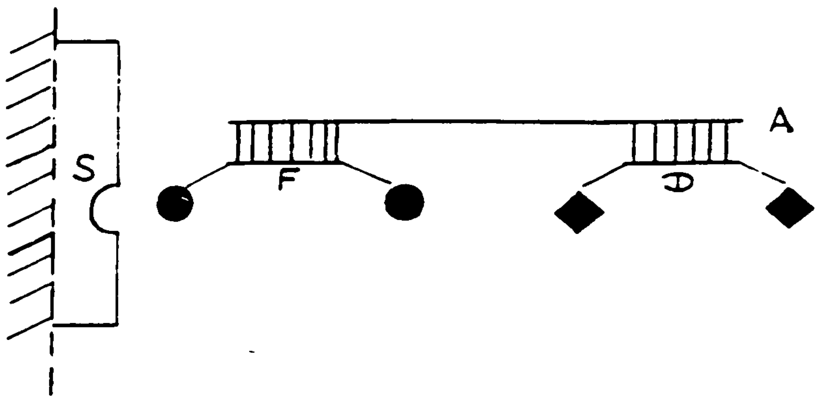
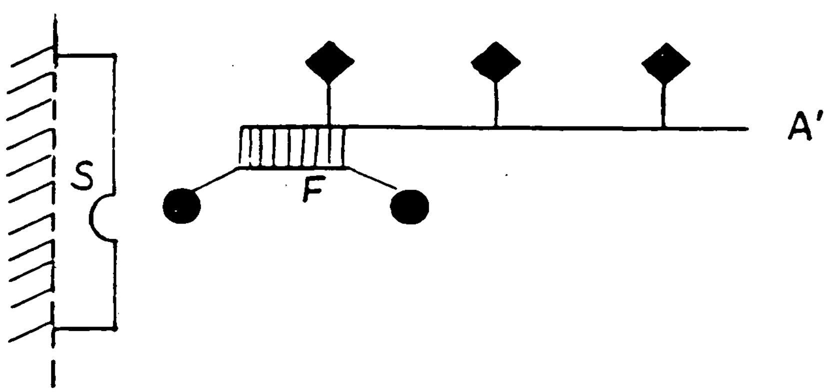


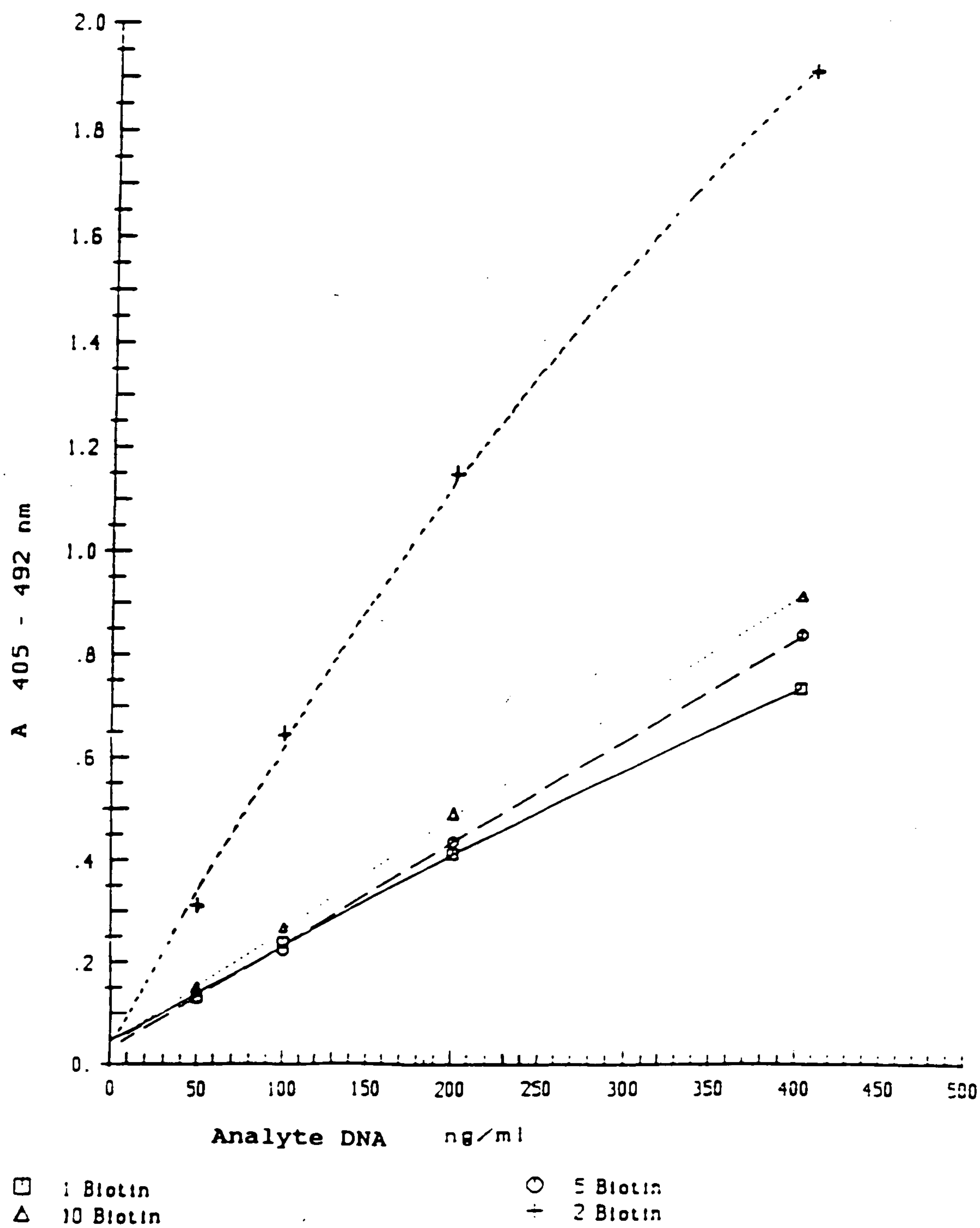
FIG. 2



= e.g. Biotin

= e.g. Digoxigenin

FIG. 3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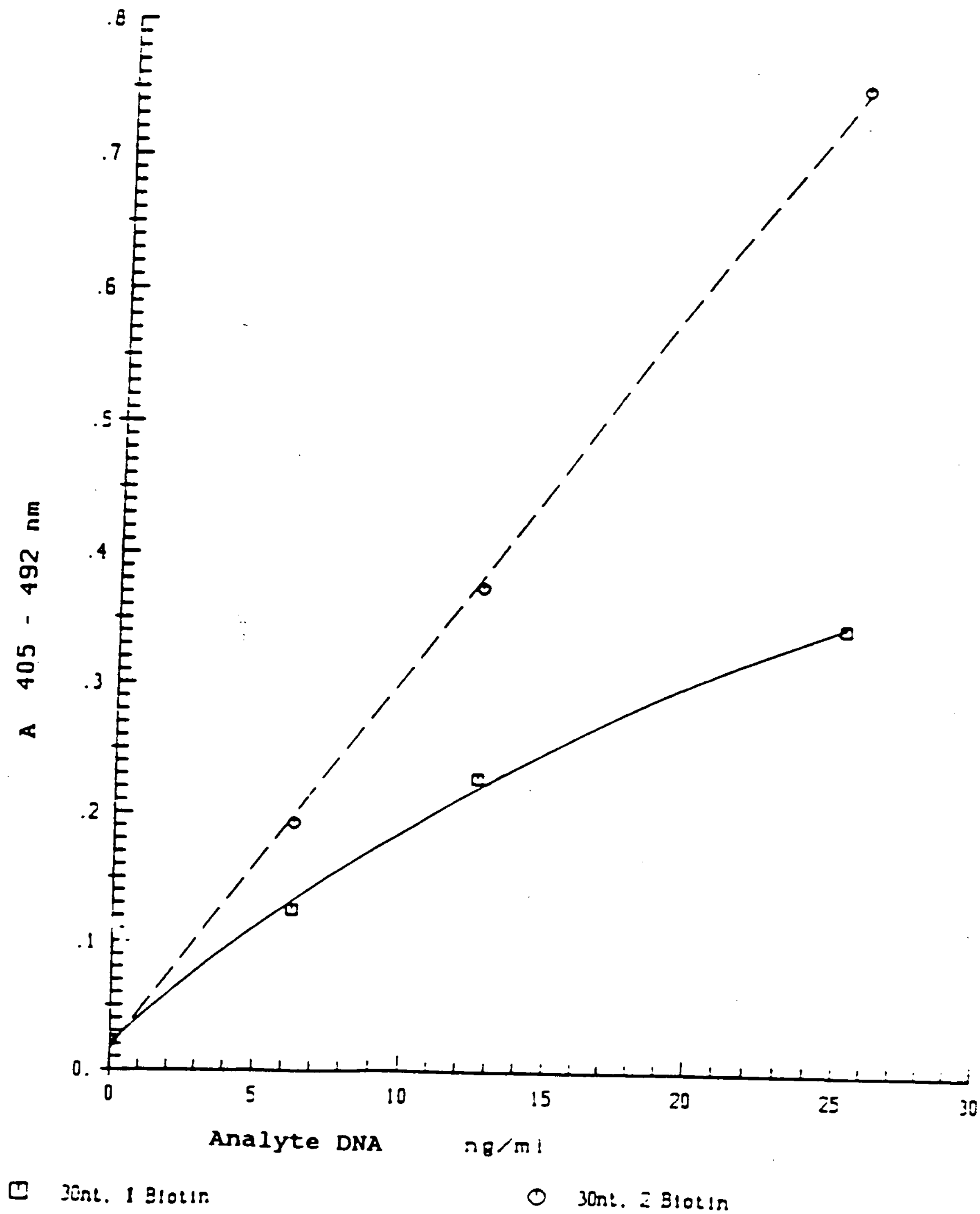
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0.
A 405 - 492 nm
0
50
100
150
200
250
300
350
400
450
500
Analyte DNA ng/ml
1 Biotin
10 Biotin
5 Biotin
2 Biotin

FIG. 4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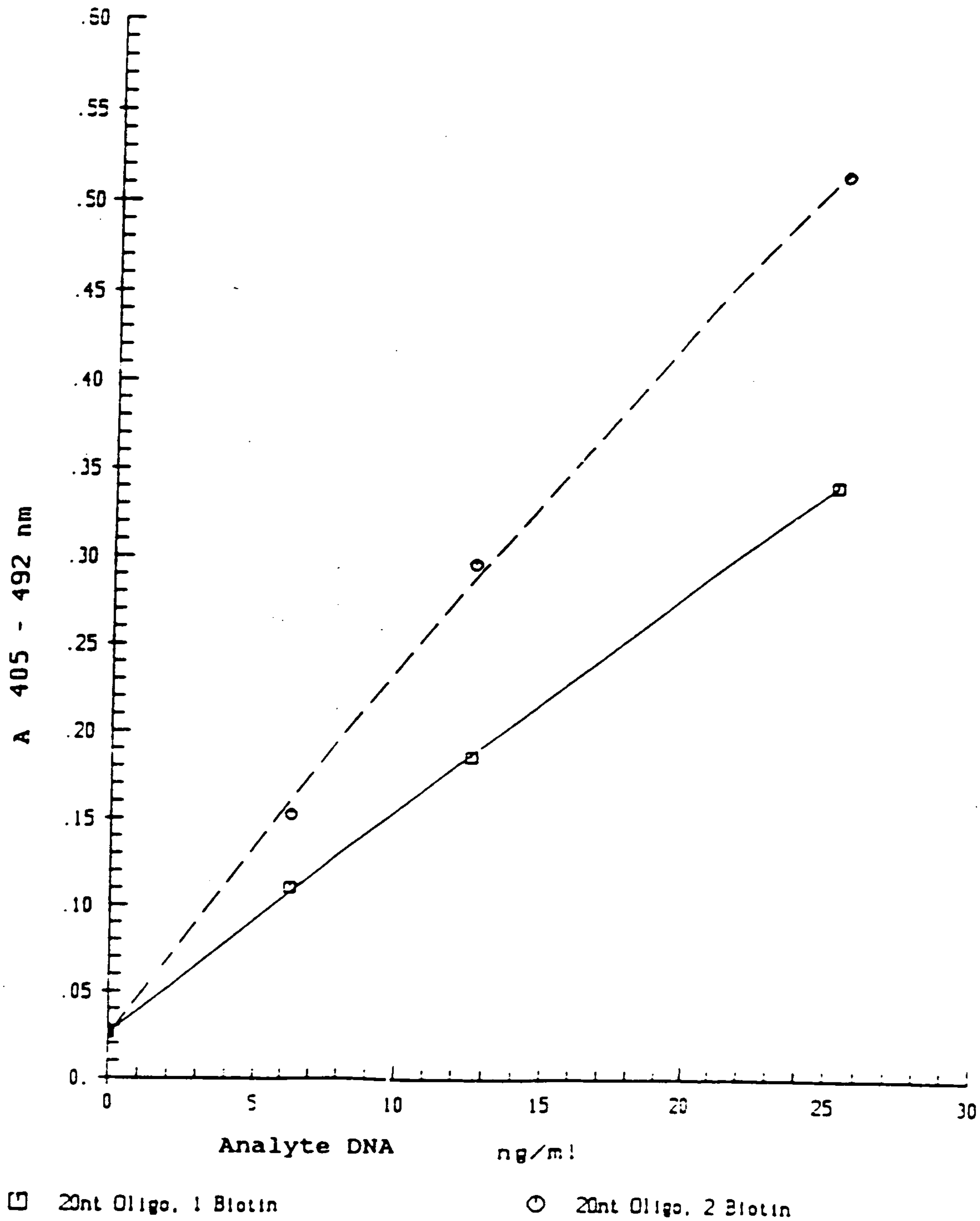
.7
.6
.5
.4
.3
.2
.1
0.
A 405 - 492 nm
0
5
10
15
20
25
30
Analyte DNA ng/ml
30nt. 1 Biotin
30nt. 2 Biotin

FIG. 5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.10
.05
0.
A 405 - 492 nm
0
5
10
15
20
25
30
Analyte DNA ng/ml
20nt Oligo. 1 Biotin
20nt Oligo. 2 Biotin

FIG. 6

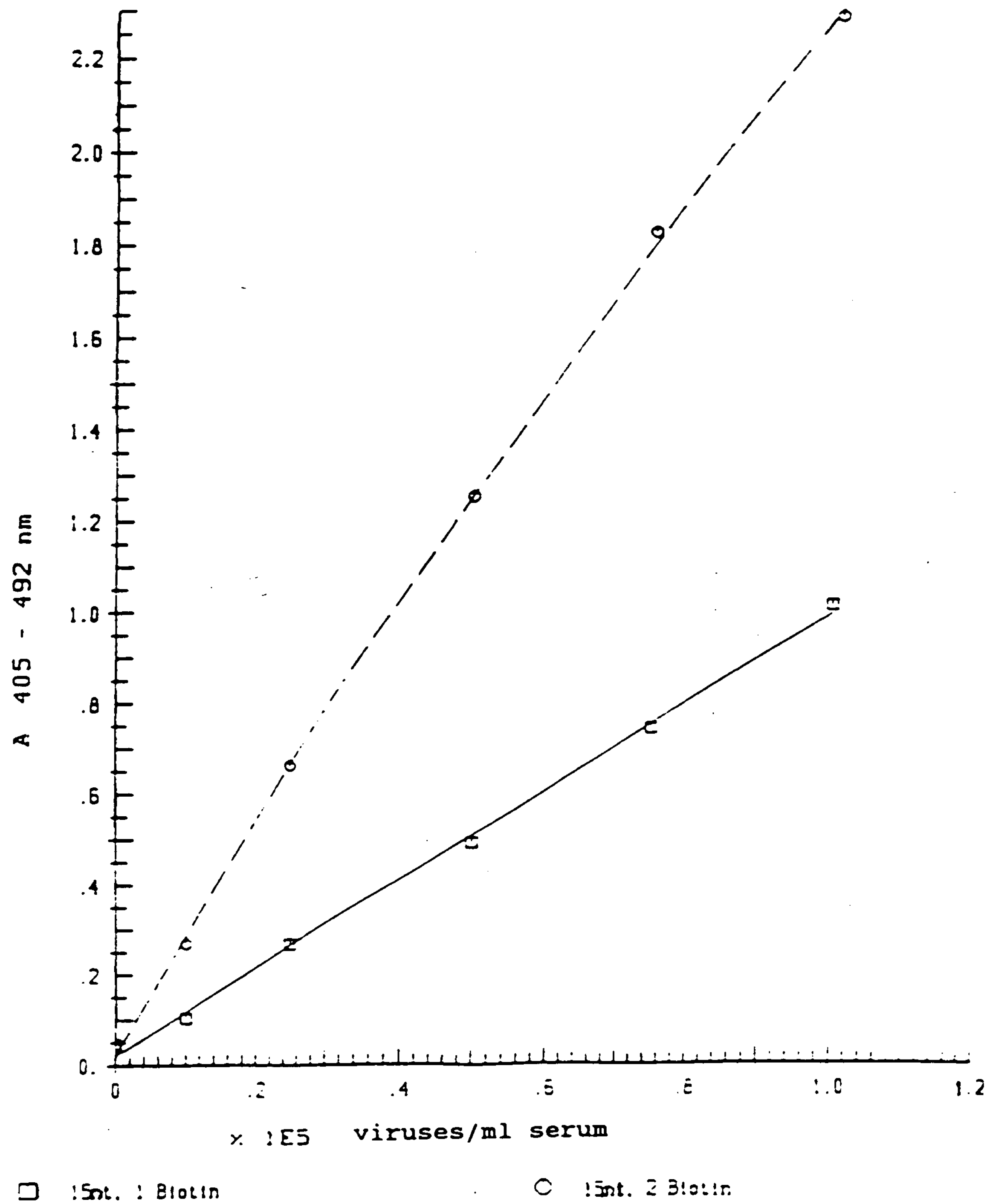
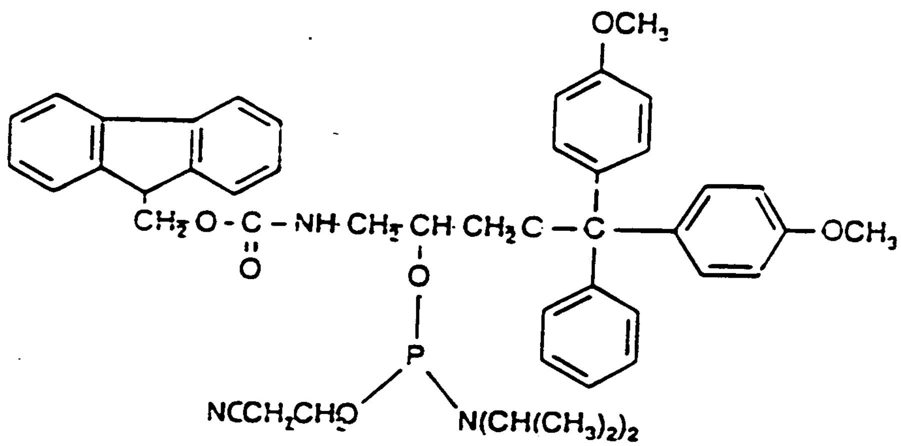

2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0.
A 405 - 492 nm
0
.2
.4
.6
.8
1.0
1.2
x 1E5 viruses/ml serum
15nt. 1 Biotin
15nt. 2 Biotin

FIG. 7



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PATENTS ACT 1990

COMMONWEALTH OF AUSTRALIA

NOTICE OF ENTITLEMENT

We, **BOEHRINGER MANNHEIM GmbH**
the Applicant/Nominated Person in respect of Application No. 19534/92 state the following:

> The Nominated Person is entitled to the grant of a patent because the Nominated Person would, on the grant of a patent for the invention to the inventors Sibylle BERNER, Stefanie KOEHLER, Cornelia KRUSE-MUELLER and Rudolf SEIBL, be entitled to have the patent assigned to it.
>
> The Nominated Person for the grant of a patent is the applicant of the basic application listed on the Patent Request Form. The basic application listed on the Patent Request Form is the first application made in a convention country in respect of the invention.

DATED this 3rd day of June, 1993

a member of the firm of
DAVIES COLLISON CAVE
and on behalf of the applicant(s)

(DCC Ref: 1510370)

930603,q:\oper\ejh,19534.084,5

AUSTRALIA

PATENTS ACT 1990

PATENT REQUEST : STANDARD PATENT

I/We being the person(s) identified below as the Applicant(s), request the grant of a patent to the person(s) identified below as the Nominated Person(s), for an invention described in the accompanying standard complete specification.

Full application details follow:

[71/70] Applicant(s)/Nominated Person(s):

Boehringer Mannheim GmbH

of

6800 Mannheim 31, Germany

[54] Invention Title:

Immobilization of nucleic acids

[72] Name(s) of actual inventor(s):

Sibylle BERNER
Stefanie KOEHLER
Cornelia KRUSE-MUELLER
Rudolf SEIBL

[74] Address for service in Australia:

DAVIES COLLISON CAVE, Patent Attorneys, 1 Little Collins Street, Melbourne, Victoria, Australia. Attorney Code: DM

Basic Convention Application(s) Details:

[31] Application Number	[33] Country	Code	[32] Date of Application
P 41 23 540.1	Germany	DE	16 July 1991

DATED this NINTH day of JULY 1992

Keith Hale

a member of the firm of DAVIES COLLISON CAVE for and on behalf of the applicant(s)